# Establishment of Genetic Linkage by Allele-specific Polymerase Chain Reaction: Application to the Lignin Peroxidase Gene Family of *Phanerochaete chrysosporium*

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**Determining linkage is problematic for genes lacking easily identifiable phenotypes and for organisms without well-defined genetic recombination systems. *Phanerochaete chrysosporium* with its lignin peroxidase (LiP) gene family typifies these difficulties. We describe an experimental approach whereby the segregation of specific alleles is directly monitored during sexual fruiting. The method establishes linkage relationships among genes for which there are no mutations, and it is applicable to a wide range of genes, gene families and organisms. Using this approach, five *P. chrysosporium* linkage groups were identified. Ten LiP genes were distributed among three of these groups. One co-segregating group contained eight closely linked LiP genes. Another LiP gene was linked to a cellobiohydrolase gene cluster. These genetic linkages were consistent with physical mapping by pulsed field gel electrophoresis. Based on the identification of allelic relationships, a uniform nomenclature for LiP genes is also described.**

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Recombination analysis is widely used to generate genetic maps, and in fungi such as *Aspertgillus nidulans, Neurospora crassa* and *Saccharomyces cerevisiae,* extremely detailed linkage maps have been constructed by meiotic segregation analysis. In recent years, these maps have included restriction fragment length polymorphisms (RFLPs) and random amplified polymorphic DNA (RAPD) as markers. However, many organisms lack well-defined systems for determining linkage, and mapping remains diffcult or impossible for genes lacking easily recognizable phenotypes.

*Phanerochaete chrysosponum,* a lignin-degrading basidiomycete with considerable biotechnical potential (for reviews see references 1 and 2), typifies these problems. Meiotic recombination analysis in *P. chrysosporium* has been complicated by a poorly defined mating system[3,4], and genetic crosses have been limited to a few simple nutritional markers". The main genes of interest, those involved in the degradation of lignocellulose and organopollutants, cannot be mapped by conventional techniques. These include genes encoding lignin peroxidases (LiPs), glyoxal oxidase (GLOX) and cellobiohydrolases (CBHs) (for reviews see references 7-9). Multiple LiP and CBH isozymes are encoded by families of structurally similar genes which exhibit complex patterns of regulation. Attempts to map LiP genes by RFLP analysis were partly successful, in that linkage was detected among some LiP-like sequences[10].

The existence of allelic variants has complicated the identification of LiP genes, but analysis of single basidiospore cultures has allowed alleles to be differentiated from closely-related genes. In the widely used *P. chrysosporium* strain BKM-1767, single basidiospores contain two identical haploid nuclei which are the products of meiosis[3]. These haploid segregants can be directly analyzed for the presence of specific alleles by restriction polymorphisms" or by allele-specific probes".

Limited physical mapping of the *P. chrysosporium* genome has been accomplished by 'walking' in genomic libraries and by pulsed field electrophoresis. Analysis of cosmid and λ libraries identified clusters of 3 LiP genes[13,14] and 3 CBH genes[15]. Southern blots of pulsed field gels localized LiP, CBH and GLOX genes to 4-5, 4, and 2 hybridization bands, respectively[16-18]. Interpreting pulsed field gels has been complicated by chromosome length polymorphisms, and by the possibility of co-migrating chromosomes. This method lacks resolution, but it has the advantage of rapidly and conveniently Idealizing cloned genes to putative chromosomes.

We report here a novel strategy for genetic mapping by segregation analysis. We systematically identified nucleotide sequence divergence among allelic pairs of all known LiP genes as well as GLOX and two CBH genes. Allelic segregation was monitored by PCR amplification of genomic DNA from single basidiospore cultures and then probing with allele-specific oligonucleotides. Owing to the dikaryotic nuclear condition and homothallic fruiting of *P. chrysosporium* strain BKM-F-1767, it was not necessary to cross strains; one set of single basidiospore progeny was adequate for all analyses. Five linkage groups were identified, one of which contained 8 closely linked LiP genes. One LiP gene, unlinked to the others, co-segregated with a CBH 1 gene cluster.

## Results

**Probe design.** To prepare allele-specific oligonucleotide probes, allelic pairs were PCR amplified with gene-specific primers, subcloned, sequenced and compared. The gene-specific primers were designed on the basis of published sequences or database depositions (Table 1). Using these primers, genomic DNA of 5 separate homokaryotic cultures was PCR amplified, cloned, partially sequenced and compared. Assuming 1:1 Mendelian segregation, it was highly probable (~95%) that each allele should be represented at least once among the 5 cultures. This was confirmed; two highly similar, but distinct, sequences were consistently observed.

The nucleotide sequences of allelic homologues were more highly conserved in coding regions relative to flanking, untranslated regions and to introns. Sequence divergence within coding regions was typically in the third position of codons, so that the predicted amino acid sequence was rarely affected. Mismatches were most commonly T-C transitions. Only one major sequence difference was detected; a 1745 bp insertion immediately adjacent to the fourth intron of a *lipI* allele. Outside this discrete insertion, however, the *lipI* alleles exhibited intense sequence homology as observed in all other allelic pairs.

Two to three hundred base pairs of sequence were adequate

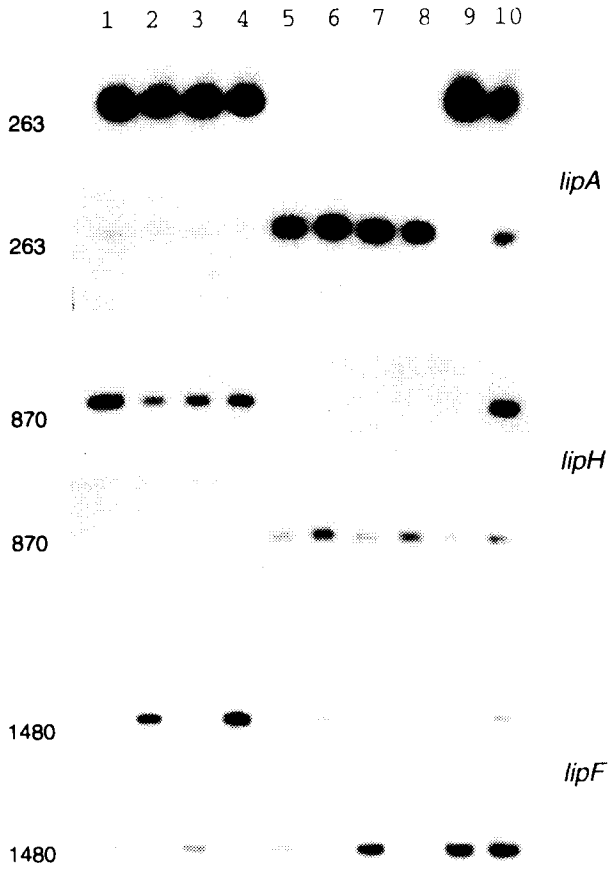


**FIGURE 1. Segregation of 3 LiP genes in selected basidiospore cultures. Genomic DNA derived from homokaryotic cultures (lanes 1-9) and from the parental dikaryon (lane 10) was PCR amplified with primers shown in Table 1. PCR products were size fractionated on agarose gels, bf-directionally blotted to Nytran and probed with allele-specific oligonucleotides (Table 1). Sizes in bp are indicated on left margin.**

to design allele-specific 17 mer probes (Table 1). In several instances, e.g. *lipE,* multiple mismatches were included in the differentiating probes. Not unexpectedly, the targets for such probes tended to be within introns. In addition to designing probes, the systematic identification of allelic relationships facilitated the development of a uniform nomenclature for LiP genes (Table 2, column 1; discussed below).

Segregation analysis. To determine genetic linkage, the segregation of specific alleles was monitored among homokaryotic segregants. The strategy involved isolation and identification of homokaryotic single basidiospore cultures, rapid extraction of genomic DNA, PCR amplification of genes using primers in Table 1. and differentiation of allelic alternatives with $^{32}$P-labeled oligonucleotide probes. Linkage was then computed from allelic co-segregation frequencies.

To illustrate the process, analysis of 9 selected single basidiospore cultures is shown (Fig. 1). Three genes, *lipA, lipH,* and *lipF,* were PCR amplified and probed with their corresponding oligonucleotides (Table 1). In the case of two closely-linked genes, *lipA* and *lipH,* the pattern of hybridization is very similar except for 1 of the 9 basidiospore cultures shown (lane 9). In contrast, the hybridization pattrern for an unlinked gene *(lipF)* is quite different illustrating the random segregation of alleles. As expected, PCR products from the parental dikaryotic culture (lane 10) hybridized to all probes.

The 12 P. *chrysosporium* genes analyzed were assigned to 5 linkage groups (Table 3). Eight of the LiP genes were tightly linked. Five of these, *lipA. lipB, lipC, lipI,* and *lipJ,* were previously shown to hybridize to two distinct hands on Southern blots of Clamped Homogeneous Electrical Field (CHEF) gels[13]. One interpretation, now confirmed, had been that the two bands represented chromosomal homologues corresponding to a single linkage group. Of the three new genes *(lipE, lipG, lipH)* mapped to this same linkage group, *lipE* may be most significant becuase of its high expression levels[19,20]. Within this same linkage group, recombination was detected between *lipA* and *lipC.* This was somewhat unexpected because physical maps show *lipA* immediately adjacent to *lipB* and only 15 kb from *lipC*[13,14]. Similar discrepancies between physical and genetic maps have been documented (for recent example see reference 21).

The genes *lipD and lipF,* were unlinked to all other LiP genes. Previously[17,18] *lipD* and *cbh1-3* were localized to a large dimorphic chromosome of approximately 4.4 and 4.8 mb. The segregation data is entirely consistent with these earlier CHEF gel blots: *lipD* is unlinked to all other known LiP genes but linked to *cbh1-3.* Recently, D'Souza et al. [16] reported *lipF* hybridizing to a relatively small chromosomal band on Transverse Alternating Field Electrophoresis (TAFE) gels. A similar hybridization result was obtained by CHEF gel blotting (data not shown). An integrated physical and genetic map is shown in Figure 2.

## Discussion

Overall, a relationship between genomic organization and transcriptional regulation in *P. chrysosporium* is not obvious. Numerous studies have shown that LiP gene expression is derepressed under nutrient limitation and that certain genes respond differentially to carbon versus nitrogen starvation. Transcript

**TABLE 1. Gene-specific PCR primers and allele-differentiating probes.**

| Gene[1] | 5′-primer | 3′-primer | length(bp)[2] | Probes[3] |
|---|---|---|---|---|
| *lipA* | GAATTCTGCAGTAGAGTGGCTGCTG | GAATTCCTAGTAAAGCCGAAGTTCC | 263 | TTTCAGGAAATG(C/T)AATC |
| *lipC* | ATGGCCTTCAAGAAGCTCCTTG | GAAGTTGGTCTCGATCTCGT | 480 | TCCG(C/T)GCTGC(G/T)CAGGGT |
| *lipD* | CCGGTCTCAGCAGCAGCA | GTACGTGGTCTCGATCGAGG | 440 | CGATCAT(T/C)ACCTTCTCC |
| *lipE* | CCGAACCGGACATGGCCTTC | TGCAAGAATTTAGGCCTTGT | 1535 | (T/C)GGTA(T/C)G(T/C)(A/C)CGGT TC(C/T)T |
| *lipF* | TCTCGATCCAGGCTGCCCAA | AAGTACTTACATGCGCTGCA | 1480 | TGCGCAT(A/G)AGTTAGCCA |
| *lipG* | CTACCAAGGCTGCTCCCGAT | CGGGAGGGTCGTGAGCGACG | 560 | CGTCTCATTACT(A/G)ACTA |
| *lipH* | CTTATTCCAAGGCGCTCAGT | GCCCGGGAGCGGAGATTCGA | 870 | CT(T/C)ACCCAG(A/T)CGTAGCG |
| *lipI* | TATATGCCTCTGAGCTCCTG | ACGTCTGTCTAGAAGTATGC | 473 or 2217 | |
| *lipJ* | GCCATCGCGATCTCTCCC | GACAAAGAATTGCGTATC | 675 | ATGCCTACGCA(C/T)AT(G/A)GG |
| *glx* | TCACACCTTCGCTCTACACG | TATTTACTCCAGGGTCGGCG | 680 | CGAGAC(C/T)CTCGACCCGC |
| *cbh1-3* | GCTAAGTACGGTACCGGCTA | GTGACAACGGTGAAGGGCTT | 330 | CCGCATGGG(C/T)GACCAGA |
| *cbh1-4* | CAACAGGCTGGCACCAACAC | TGCCAGTAACCAGTTCCGTA | 508 | CGGC(G/A)GAGAACCA(C/T)CCC |

[1]Lignin peroxidase (LiP) synonyms are listed in Table 2. *glx* and *cbh1-3* encode GLOX[23] and CBH1[15], respectively. Cosmid and λ mapping show *lipA* and *lipB* immediately adjacent [13,14], and so one set of primers was used to score the pair. [2]Approximate PCR product length for both alleles. A 1745 bp insertion was detected within the *lipI2* allele. [3]For each gene, a pair of oligonucleotide probes were synthesized to differentiate the two alleles. This pair differed by one *lipA, lipD, lipF, lipG, glx, cbh1-3).* two *(lipC, lipH, lipJ)* or four *(lipE)* bases. The substantial differnce in *lipI* allele lengths obviated the need for probing.

levels of 6 LiP genes have been studied in both C- and N-starved cultures. Transcript levels of *lipA, lipB,* and *lipI* differ substantially from each other but remain unchanged in C- versus N-starved cultures[18,22]. In contrast, *lipC* and *lipJ* mRNAs arc at least 100-fold more abundant in N-starved media relative to C-starved media[18]. The *lipD* gene, and to a lesser extent *lipE,* are preferentially expressed in C-starved media[19,22]. Glyoxal oxidase, coordinately expressed with the *lipA* in C- and N- starved cultures[18,23], is not linked to *lipA* (Fig. 2).

Independent segregation by *lipD* and *lipF* may reflect physiological and evolutionary significance. Expression of *lipD* is dramatically increased by carbon limitation[18,22,24], and it may be relevant that *lipD* is linked to *cbhl-1* and *cbhl-2,* cellobiohydrolase genes also derepressed by carbon limitation". Regulation of the other unlinked LiP gene, *lipF,* has not been thoroughly investigated. Interestingly, when intron/exon structure is used as the criteria for delineating LiP genes into four *groups,lipD* and *lipF* arc assigned separate subfamilies[7,20]. Clustal analyses[25] of deduced LiP amino acid sequences clearly show *lipD* to be distinct from *lipF* and the other LiP genes.

Like other complex gene families, considerable uncertainty exists as to the total number and structure of LiP genes. For example, identical upstream regions were recently reported in six LiP "genes''[26], but our analysis shows that these six clones are actually the same gene or allelic variants thereof. The number of lignin peroxidase genes in *P. chrysosporium* has been variously set from 5 (ref. 16) to 15 (ref. 26). Contributing to this confusion are the widespread use of two distinct laboratory strains (BKM-F-1767 and ME446), the presence of allelic variants within these dikaryotic strains, the publication of partial sequences with or without database deposition, inconsistent nomenclature for clones/genes, and separate publication of highly similar or identical sequences. In the process of mapping the LiP genes, allelic relationships were established. On that basis, we include a uniform nomenclature which, if adopted by other researchers, may reduce confusion in the future (Table 2). The system arbitrarily employs letters to designate genes and numbers to assign alleles (Table 2; column 1). This system makes no attempt to assign specific isozymes to these genes. A working assumption is that strains feature two alleles of each gene; these are labeled 1 and 2 for BKM-1767 alleles, 3 and 4 for ME446 alleles. New genes and alleles can be designated with alphabetical and numerical suffixes, respectively.

The mapping strategy described here is generally applicable to cloned DNA from any organism from which haploid products of meiosis are available. For basidiomycetes such as *P. chrysosporium,* crosses and parental strains are unnecessary. Only homokaryotic basidiospores are required. Many fungi, although unable to fruit in culture, produce abundant viable basidiospores in nature. Using the approach described here, basidiocarps collected from the field or herbarium samples could provide the spores needed to construct genetic linkage maps. Other orga-

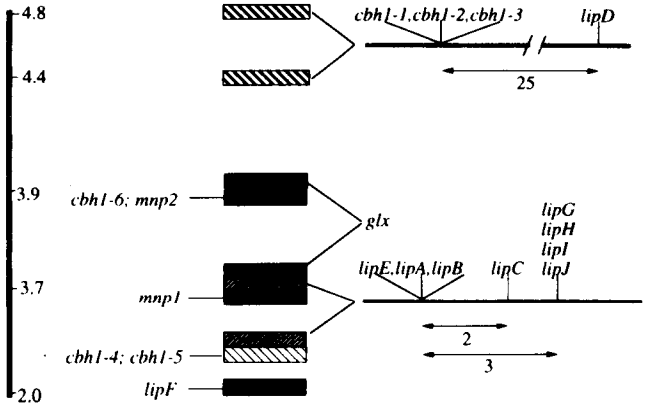


**FIGURE 2. Integrated physical and genetic linkage map of 12 *P. chrysosporium* genes. Approximate CHEF migration pattern of chromosomes is schematically represented with size estimates in mb on left vertical bar. Homologous chromosome pairs have identical shading. Thick horizontal bare represent linkage results. Percent racombination is indicated under arrows. The relative order of *lipG, lipH, lipl,* and *lipJ* is unknown. The order of *cbh1-1, cbh1-2,* and *cbh1-3* was established by cosmid mapping[15], but the position of *lipD* relative to the *cbh1* cluster is uncertain. Chromosome assignments for manganese peroxidases *(mnps)* and two cellobiohydrolases *(cbh1-5, cbh1-6)* are based solely on CHEF gel blots[17,33].**

**TABLE 2. Current list of lignin peroxidase genes.**

| Designation[1] | Source | Synonyms[2] | Reference | Accession Number |
|---|---|---|---|---|
| *lipA1* | BKM-1767 | ML1(c) | 22, 34 | Y00262; M21913 |
| | | H8 (g) | 11, 35 | X06689 |
| | | LPOA(g) | 14, 36 | M22720 |
| *lipA2* | BKM-1767 | LiPA(g) | 12, 13 | X54256 |
| | | ML4 | 37 | M27884 |
| *lipA3* | ME446 | LIG2(g) | 38 | none |
| *lipA4* | ME446 | LG1(g) | 39 | none |
| *lipB1* | BKM-1767 | none | 13 | X54257 |
| *lipB2* | BKM-1767 | LPOB(g) | 14 | M37701 |
| *lipB3* | ME446 | LIG3(g) | 38 | none |
| *lipC1* | BKM-1767 | CLG5(c) | 40, 41 | M18794 |
| | | GLG5(g) | 13 | X55343 |
| *lipC2* | BKM1767 | LiP6(g) | 42 | M63496 |
| *lipD1* | BKM1767 | CLG4(c) | 40, 41 | M18743 |
| *lipD2* | BKM-1767 | LiP2(g) | | X15599 |
| *lipE1* | BKM-1767 | LPO811(c) | 19 | L08963 |
| *lipE3* | OGC101 | L18(c) | 43 | M74229 |
| | | LG2(g) | 20 | M92644 |
| *lipF1* | BKM-1767 | GLG6(c)(g) | 44 | M80213; M77508 |
| *lipG1* | BKM-1767 | ML-5(c) | 37 | none |
| *lipH3* | ME446 | LIG1(g) | 45 | M24082 |
| *lipI1* | BKM-1767 | O282(g) | 11 | none |
| *lipJ1* | BKM-1767 | V4(g) | 11 | none |
| *lipJ3* | ME446 | LIG4(g) | 38 | none |

[1]Designation systematics explained in text. [2]Parenthesis indicate type of DNA clone: (c), cDNA; and (g), genomic.

**TABLE 3. Segregation of *P. chrysosporium* alleles.**

| | Percent Co-segregation[1] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | *lipC* | *lipD* | *lipE* | *lipF* | *lipG* | *lipH* | *lipI* | *lipJ* | *glx* | *cbh1-3* | *cbh1-4* |
| *lipA/B* | **98(65)** | 53(64) | **100(66)** | 58(43) | **97(69)** | **97(69)** | **97(69)** | **97(69)** | 55(42) | 53(66) | 51(57) |
| *lipC* | | 52(60) | **98(62)** | 56(41) | **98(65)** | **98(65)** | **98(65)** | **98(65)** | 52(40) | 57(61) | 53(53) |
| *lipD* | | | 51(61) | 64(42) | 50(64) | 50(64) | 52(64) | 52(64) | 54(41) | **75(61)** | 64(53) |
| *lipE* | | | | 59(41) | **97(67)** | **97(67)** | **97(67)** | **97(67)** | 55(42) | 56(64) | 56(54) |
| *lipF* | | | | | 58(43) | 58(43) | 58(43) | 58(43) | 58(24) | 51(41) | 59(37) |
| *lipG* | | | | | | **100(70)** | **100(70)** | **100(70)** | 52(42) | 58(66) | 51(57) |
| *lipH* | | | | | | | **100(70)** | **100(70)** | 52(42) | 58(66) | 51(58) |
| *lipI* | | | | | | | | **100(70)** | 52(42) | 58(66) | 51(58) |
| *lipJ* | | | | | | | | | 52(42) | 59(66) | 51(57) |
| *glx* | | | | | | | | | | 63(41) | 52(40) |
| *cbh1-3* | | | | | | | | | | | 51(53) |

[1]Percentage of co-segregating alleles for all pairwise comparisons of genes. The number of progeny analyzed follows in parentheses. Numbers in bold type indicate statistically significant (P=.01) linkage by $\chi^2$ analysis.

nisms in which haploid products of meiosis are readily available include a number of important conifer species. For example, Tulsieram et al.[27] exploited this characteristic to construct a RAPD map of white spruce.

The method can be modified to suit a variety of experimental situations. The targeted markers need not be specific structural genes. Degenerate primers amplifying highly conserved genes. or random PCR products could also be used, Allele-specific probes could be tagged with fluorescent dyes, or perhaps eliminated altogether by choosing suitably selective PCR conditions (for example see references 28 and 29). Finally, the number of PCR reactions might be substantially t-educed by simultaneously amplifying several genes of varying lengths.

## Experimental Protocol

**Strains.** P. chrysosporium strain BKM-F-1767was obtained from the Center of Forest Mycology Research, Forest Product Laboratory, Madison, Wisconsin and used throughout the study. Following fruiting[30], germinating single basidiospores were isolated from agar plates with a needle and a dissecting microscope.

DNA purification and amplification. Total genomic DNA was extracted directly from 15 mm agar plugs as described[31]. The final pellet was resuspended in 100 to 500 µl TE and diluted 50-fold prior to use. A single extract is stable for years at 4 °C and provides enough material for thousands of PCR amplifications. All PCR reactions were performed in 50 µl reactions with the following buffer: 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, 0.2 mM each dNTP, 10.5 pmol of each primer, and 1.25 units Tag polymerase. One to five µl of diluted genomic DNA was subjected to an initial cycle of denaturation (6 min, 94 °C), annealing (2 min, 54°C), and prolonged extension (40 min. 72°C) followed by 35 cycles of denaturation (1 min, 94°C), annealing (2 min. 54°C) and extension (5 min, 72°C). A final 15 min extension at 72°C was also included.

**Southern blots.** Following amplification, larger PCR products ( > 400 bp) were size fractionated on 1-2% Seakem GTG agarose (FMC Rockfand, ME) gels. Smaller products were electrophoresed in gels containing 2% SeaKemGTG plus 1% Nuseive agarose (FMC). Gels were bidirectionally blotted to Nytran (Schleicher and Schuell, Keene, New Hampshire) according to the manufacturer's recommendations and then UV cross-linked (UV Stratalinker 1800, Strafagene Inc., La Jolla, CA). Blot hybridization conditions were as previously described[12,32]. Generally, this involved hybridization and washes in 6X SSC at temperatures of $T_d$-3°C, where $T_d$=4(G + C) +2(A + T). Additional washes at slightly elevated temperature or lowered salt concentrations were occasionally used to improve resolution of probes.

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